Regular Paper

PAPILLOMATOSIS IN CATTLE: *IN SITU* DETECTION OF BOVINE PAPILLOMAVIRUS DNA SEQUENCES IN REPRODUCTIVE TISSUES

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ABSTRACT

Bovine papillomatosis is a common viral infection in Brazil that is caused by a bovine papillomavirus (BPV). Dissemination is by direct contact between infected animals, although the investigation of other modes of transmission is a very important aspect in the management of this condition. BPV DNA sequences have been detected in many tissues by using the polymerase chain reaction. In this work, we used *in situ* hybridization to detect BPV DNA sequences in bovine reproductive tissues and cells. The detection of BPV in these tissues strongly suggests that these sequences could be an important alternative of viral transmission that could contribute to the widespread incidence of bovine papillomatosis and its complex pathology. Alternatively, the viral sequences could result from cell apoptosis and may therefore not be directly involved in the infection.

Key words: Bovine papillomavirus, *in situ* hybridization, papillomatosis

INTRODUCTION

Papillomaviruses are epitheliotropic and mucosotropic double-stranded DNA viruses that infect humans and cattle and, when associated with co-factors, can cause cancer and death. In cattle, benign lesions caused by this virus may become malignant and, in humans, cervical tumors related to these viruses are one of major causes of death in women [2].

Infection by bovine papillomavirus (BPV) causes cutaneous papillomatosis and benign proliferative lesions that can result in severe injuries and losses in animal production [7]. The lesions can regress spontaneously over a period of 6-12 months and most of the affected animals are usually less than 2 years old. Animals previously affected by papillomatosis and exposed to co-factors, such as chronic consumption of the bracken fern *Pteridium aquilinum*, often develop neoplastic lesions, including bladder cancer (identified by clinical symptoms such as chronic enzootic hematuria, CEH) [3] and esophageal carcinoma [1,4].

Six types of BPV have been identified: BPV1, BPV2 and BPV5 are related to cutaneous fibropapillomas, whereas BPV3, BPV4 and BPV6 are related to squamous papillomas of the skin and esophagus [2]. In addition, Ogawa *et al.* [10] have discussed new putative BPV types and possible subclinical infection by these viruses.

In 1988, we demonstrated a significant increase in the level of chromosomal lesions in peripheral lymphocytes of animals affected by CEH and chronically exposed to bracken fern [13]. To distinguish the effects of the virus from those of bracken, we examined the clastogenic responses in the peripheral lymphocytes of animals inoculated with samples of bovine blood known to contain BPV DNA sequences. Stocco dos Santos *et al.* [11] reported a significant increase in the level of chromosomal aberrations in short-term cultures of blood lymphocytes from infected animals not exposed to bracken fern and compared to normal

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This report contains the results from a MSc dissertation and PhD thesis by Andréa Yaguiu and Claudemir de Carvalho, respectively.

controls without BPV and bracken fern. Leal *et al.* [9] subsequently found that bovine palate fibroblasts exposed to BPV4 oncoproteins and quercetin showed characteristic markers of chromosomal damage, principally in metacentric and submetacentric chromosomes, as revealed by C-banding and possible centric fusions related to telomeric alterations.

Considering the widespread occurrence of papillomatosis in livestock, we have been searching for different forms of BPV transmission to explain this dissemination. Carvalho *et al.* [5] and Yaguiu *et al.* [14] reported the presence of BPV DNA sequences in bovine reproductive tissues and cells, including oocytes, ovarium, uterus, cumulus cells, uterine fluids, semen and spermatozoa. In this report, we describe the use of *in situ* hybridization to localize BPV DNA sequences in bovine warts, reproductive tissues and spermatozoa, and discuss the possibility that these sequences are not simply products derived from the lyses of infected epithelial cells.

MATERIAL AND METHODS

Animal selection

Various groups of animals were used to obtain the different samples for evaluation. These included six three-year-old females (*Bos taurus taurus*) (A1-A6), four females with cutaneous papillomatosis, one female with enzootic hematuria, one female without clinical symptoms of BPV infection, two slaughtered four-year-old females (*Bos taurus taurus)* without cutaneous warts but raised in a herd with a high incidence of papillomatosis (sA1-sA2), and two four-year-old males (*Bos taurus taurus*) that provided the semen samples and were raised in a herd with a high incidence of papillomatosis (male 1, male 2).

Sample collection

Samples were collected using appropriate precautions to avoid any contamination. All of the procedures were undertaken using disposable gloves that were changed at each step. After cleaning the skin with iodinated alcohol, horizontal slices of warts were collected by sectioning parallel on the skin surface using disposable scalpels. The samples were immediately stored on dry ice or formalin in sterile disposable flasks. Uterine samples were collected immediately after delivery and stored at 4°C until analyzed. Ovarian and uterine samples were also obtained from slaughtered cows. Fresh semen samples were collected directly in disposable sterile flasks after cleaning the penis and genital region.

Assessment of DNA amplification

To assess the ability to amplify DNA, about 100 ng of sample genomic DNA was amplified in each round of PCR for viral detection using a pair of primers specific for the bovine β–globin gene (FW: 5′-aacctctttgttcacaaccag-3' and Rev: 5'-cagatgcttaacccactgagc-3'), with the expected PCR product being 430 bp long. Amplification reactions were done in 50 mmol/l KCl, 20 mmol/l Tris-HCl (pH 8.4), 1.5 mmol/l $MgCl_2$, 200 µmol/l of each dNTP, 0.4 µmol/l primer and 1 IU of *Taq* polymerase. The PCR conditions consisted of an initial step at 94°C for 3 min, followed by 35 cycles at 94°C for 50 s, 60°C for 1 min and 72°C for 1 min, and with a final extension at 72°C for 5 min; all reactions were done in a PTC-100 thermocycler (MJ Research Inc.). The PCR products were analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide.

Semen preparation

Semen samples (0.5 ml) were thawed in a water bath at 36°C, diluted in 4.5 ml of PBS (phosphate-buffered saline) and centrifuged at 1,500 x g. The pellet was treated with 2 units of trypsin for 90 s and the residual enzymatic activity was removed using a PBS solution containing 0.4% bovine serum albumin, as proposed for the trypsin treatment of embryos [12]. The treated semen samples were centrifuged again and suspended in PBS.

DNA extraction

DNA was extracted with a DNA Easy Blood and Tissue kit (Qiagen), in accordance with the manufacturer′s instructions.

Detection of viral sequences

About 300 ng of sample DNA and complete genomes of each virus (cloned in pAT153) (positive control), in addition to the reaction control (without DNA), were amplified by PCR using specific primers for BPV1 (FW: 5'-ggagcgcctgctaactatagga-3' and RV: 5'-atctgttgtttgggtggtgac-3'), BPV2 (FW: 5'-gttataccacccaaagaagaccct-3' and RV: 5'-ctggttgcaacagctctctttctc-3') and BPV4 (FW: 5'-gctgaccttccagtcttaat-3' and RV: 5'-cagtttcaatctcctcttca-3'). These primers were complementary to the L2 (BPV1), L1 (BPV2) and E7 (BPV4) regions of the BPV genomes and amplified fragments of 301 bp, 164 bp and 170 bp, respectively. The PCR was done as described by Stocco dos Santos *et al.* [11] in a PTC-100 thermocycler using the following conditions: 300 ng of sample DNA in 50 µL was denaturated for 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 40 s and annealing at 55°C for 40 s, with a final 1 min extension at 72°C. A 1 µL aliquot of the reaction mixture was run on a 1% agarose gel.

Sequencing

The BPV1, 2 and 4 sequences were selected as positive BPV controls for the wart samples and their products were cloned using a TOPO TA Cloning® kit for sequencing

(Invitrogen). The cloned fragments were sequenced using an A.L.F. express DNA sequencer (Pharmacia Biotech). Both strands of the selected clones from each sample were sequenced using a Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing kit with 7-deaza-dGTP and universal and reverse primers.

In situ hybridization (ISH)

Before ISH, wart, uterine and ovarian samples were prepared on histological slides. *In situ* hybridization was done using biotin-labeled PCR products as probes. The probes, which were labeled using a DAKO GenPoint kit (DAKO, Carpinteria,USA), were prepared from PCR products obtained from amplification of the cloned viral genome and from PCR products obtained from the samples. The negative controls were prepared using PCR products of genes not detected in amplifications of our samples. The semen samples were prepared in 2 mmol/l dithiothreitol (DTT) to obtain decondensed sperm heads, as described by Kobayashi *et al.* [8].

RESULTS

Three types of BPV sequences were found in wart, uterine and ovarian fragments and semen samples using PCR. Initial PCR assays using β-globin-specific primers showed that the DNA from the samples was suitable for use in the amplifications. The PCR amplifications using specific primers for BPV 1, 2 and 4 yielded products with the expected sizes (summarized in Table 1 and Fig. 1). Figure 1 shows the PCR amplification products of 301 bp, 164 bp and 170 bp for BPV1, 2 and 4, respectively.

Table 1. Detection of BPV DNA sequences in the different samples.

Animal/sample	BPV type		
	BPV1	BPV2	BPV4
A1/wart	+	+	+
A2/wart	+	+	+
A3/wart	+	+	+
A4/wart	+	-	-
A5/wart	+	-	-
A6/wart	+	-	-
A2/uterus	+	+	+
A3/uterus	+	+	+
sA1/ovarium	+	+	-
sA1/uterus	+	+	-
sA2/ovarium	-	-	-
sA2/uterus	-	-	-
Male 1 semen	-	+	-
Male 2 semen	-	+	-

A1–A6: females , sA1–sA2: slaughtered females.

The same fragments obtained by PCR were used as probes for ISH. All of the samples analyzed by PCR were prepared for hybridization on slides, one for each probe (BPV1, BPV2 and BPV4), one for a negative control and β-globin as a positive control. The results are shown in Figs. 2A-E, 3A-D, 4A-D and 5, and confirmed the PCR data shown in Fig. 1. Labeling indicated that the DNA sequences selected as probes hybridized with sequences present inside the cells and not between them. Labeling for BPV2 was also seen inside spermatozoa, in agreement with the PCR results.

DISCUSSION

Papillomavirus infection starts in epithelial basal layers, with the virus undergoing differentiation throughout the activation of E (early) and L (late) genes to produce virions on the superior external layers [6]. The penetration of a non-enveloped virus into the target cell involves two steps [1]: i) attachment and interaction with the cell membrane through binding to a surface receptor molecule and ii) penetration of the viral particle. Papillomavirus probably attaches to a conserved receptor widely expressed on the cell surface. Recent studies have suggested that the entrance of papillomavirus involves interaction with cell surface molecules that act as receptors, such as integrins α6β4 or α6β1 or heparan sulfate, or mechanisms such as endocytosis via a clathrin-dependent pathway [6].

As shown above, three BPV DNA sequences were detected in a given tissue, a finding confirmed by *in situ* hybridization. Thus, the probes were detected not only inside wart cells of the infected epithelium, but

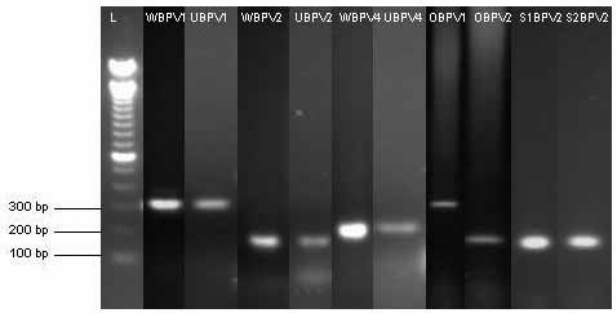

Figure 1. PCR detection of BPV1, 2 and 4 DNA sequences in wart (**1-BPV1, 3-BPV2, 5-BPV4**), uterus (**2-BPV1, 4-BPV2, 6-BPV4**), ovarium (**7-BPV1, 8-3PV2**) and semen (**9 and 10-BPV2**). **L** –100 bp adder.

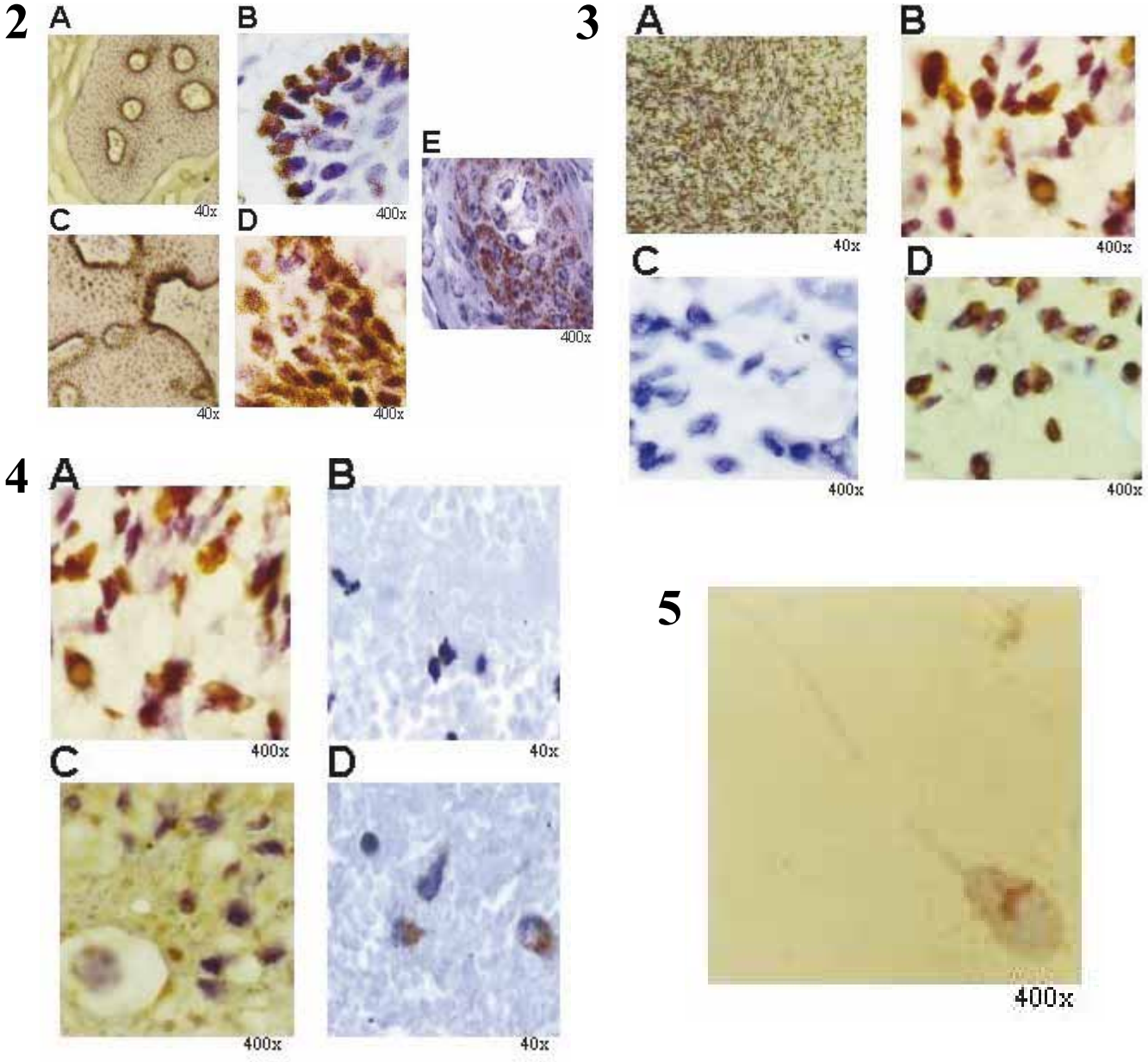

Figure 2. (A-E) *In situ* hybridization with BPV1, BPV2 and BPV4 probes in wart sections. (**A**) and (**B**), BPV1 detection (40x and 400x, respectively), (**C**) and (**D**) BPV2 detection (40x and 400x, respectively) and (**E**) BPV4 detection (400x). BPV_2 detection (5 and 50 µm, respectively) and (E) BPV4 detection (50 µm).
Figure 3. (A-D) *In situ* hybridization with BPV2, BPV4 and β-globin probes in ovarian sections: (**A**) and (**B**), hybridization with β-globin, a positive control for the hybridization procedure (Bars: 2,5 and 50 µm, respectively), (**C**) negative control with the BPV4 probe (400x), not detected by PCR and (**D**) hybridization with the BPV2 probe (400x).
Figure 4. (A-D) *In situ* hybridization in uterine sections. (**A**) positive control (β-globin probe) (400x), (**B**) BPV1 probe (40x), (**C**) BPV2 probe (400x) and (**D**) BPV4 probe (40x). Bars: A and C = 50 µm, B and D = 5 µm.
Figure 5. *In situ* hybridization with the BPV2 probe in spermatozoa (Bar = 50 µm).

also in ovaries, uterus and spermatozoa, which are not normal targets for the virus. It could be argued that the DNA sequences detected in tissues not specific for the virus resulted from the phagocytosis of viral DNA fragments carried by blood to different tissues. However, the presence of viral sequences in reproductive tissues and gametes that are incapable of phagocytosis supports the possibility of new pathways for viral transmission. This suggestions requires further investigation to assess its relevance

for dissemination of the virus. Finally, co-infection by different types of BPV (such as shown here) is a crucial aspect that requires the development of appropriate polyvalent vaccines.

ACKNOWLEDGMENTS

The authors thank Carolina da Paz Sabino for editorial support and CNPq fo the fellowships. This paper is dedicated to Prof. Dr. Carminda da Cunha-Landin.

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Received: June 13, 2006
Accepted: August 7, 2006